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Erythroid-specific expression of human β-globin genes in transgenic mice

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Transgenic mice carrying human β-globin genes were produced by microinjecting linear DNA molecules containing cloned β-globin genes with up to 4300 bp of 5′-flanking sequence and 1700 bp of 3′-flanking sequence. Most (15 of 20) of these transgenic mice expresssed the human β-globin genes in blood cells and the level of expression in some mice was comparable with that obtained from endogenous β-globin genes. Human β-globin gene expression appeared to be restricted to cells of the erythroid lineage and was first detected between 11 and 14 days of development, in parallel with mouse β-globin. Constructs with as little as 48 bp of 5′-flanking sequence also appeared to be expressed appropriately. The mRNA transcripts had correct 5′ ends and directed human β-globin synthesis in reticulocyte lysates. Human β-globin protein was detectable in mature erythrocytes from progeny of one of these mice. The frequency and extent of expression was severely depressed when the procaryotic vector DNA was not removed prior to microinjection.

Key words: β-globin genes/transgenic mice/erythroid-specific expression

Introduction

A variety of questions of fundamental interest can be addressed by introducing foreign genes into the germ line of mice by microinjection of fertilized eggs (Palmiter and Brinster, 1985). A number of genes have now been expressed in transgenic mice, including several metallothionein fusion genes (Brinster *et al.*, 1981; Palmiter *et al.*, 1982, 1983), transferrin (McKnight *et al.*, 1983), immunoglobulin light and heavy chain genes (Brinster *et al.*, 1983; Grosschedl *et al.*, 1984) and elastase genes (Swift *et al.*, 1984). In many of these cases, the tissue specificity and level of expression of the foreign genes resemble their endogenous counterparts. However, achieving appropriate expression of globin genes has been troublesome (Wagner *et al.*, 1981a; 1981b; Costantini and Lacy, 1981; Lacy *et al.*, 1983).

Expression of globin genes in transgenic mice would be especially desirable because much of the pioneering work on regulation of gene expression has centered on globin genes, because they are developmentally regulated, because a large number of natural mutations affecting globin gene expression have been identified (see reviews by Bunn *et al.*, 1977; Collins and Weissman, 1984) and because globins are among the best characterized proteins (Dickerson and Geis, 1983). Our first approach was to test a mouse β^{maj} – goat β^{c} hybrid gene, but it was not expressed in transgenic mice (T.Townes and R.Brinster, unpublished observations). Then we tried inserting an intact human β-globin gene next to a gene (metallothionein-human growth hormone) that is expressed well in transgenic mice. This construct allowed human β-globin gene expression but the tissue specificity resembled that of metallothionein genes rather than globin genes. In trying to understand what parts of the neighboring metallothionein fusion gene were important in directing the expression of the human β-globin gene, we discovered that removal of all but 500 bp of the metallothionein sequence allowed erythroid-specific expression of human β-globin (Townes *et al.*, 1985). By this rather circuitous route, we decided to test human β-globin genes on their own.

Results

Expression of human β-globin mRNA in transgenic mice

Figure 1 is a scheme, showing the five linear DNA fragments containing the human β-globin gene that were tested for expression in transgenic mice. This series of deletions had 4300, 815, 360, 122 or 48 bp of 5′-flanking sequence and a constant 1700 bp of 3′-flanking sequence. All of the promoter elements known to be necessary for expression of β-globin genes when transfected into tissue culture cells are included in the first four of these constructs but the last one deletes most of the elements thought to be essential for a normal level of β-globin gene expression (Dierks *et al.*, 1983). The DNA fragments shown in Figure 1 were separated from the plasmid vector sequences prior to microinjection. The microinjected eggs were allowed to develop in pseudopregnant recipients and pups were analysed for retention of the microinjected DNA by dot hybridization of nucleic acids isolated from tail biopsies. The probe used was a 287-bp *Dde*I fragment that corresponds to the 3′-untranslated region of the human β-globin gene and does not cross-react with the mouse genome. To ascertain whether human β-globin genes were expressed, transgenic mice in each group were treated with phenylhydrazine to make them anemic and total nucleic acids were isolated from samples of blood (40–50% reticulocytes) as well as other tissues (Table IA).

Human β-globin mRNA was assayed by incubating total nucleic acids with a 5′ end-labeled, 21-base oligonucleotide complementary to a region of exon 1 that differs in five positions between human and mouse (see lower portion of Figure 1). Hybrids were detected by treatment with S1 nuclease, precipitation of the protected probe with trichloroacetic acid, collection of the precipitates on glass fiber filters and scintillation counting. For quantitation, a standard curve was constructed with single-stranded M13 DNA carrying sequences corresponding to human β-globin mRNA. Figure 2 illustrates a typical standard curve and the experimental results from three transgenic mice; one, 105-7, that had no human β-globin mRNA, and two, 103-8 and 107-11, that had low and moderate levels, respectively. Note

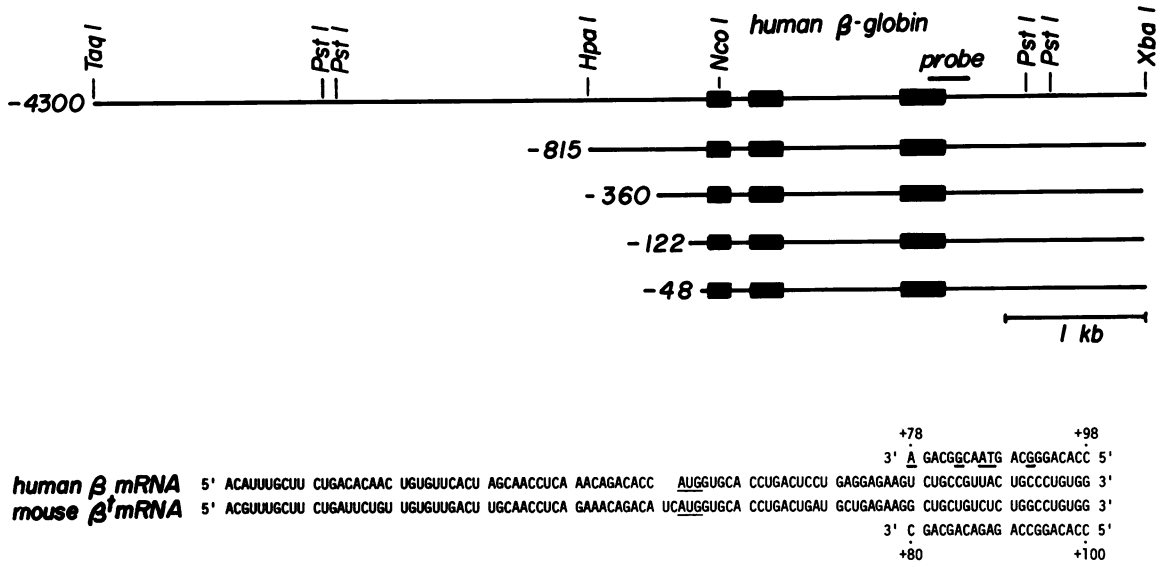

Fig. 1. Scheme showing the human β-globin gene constructs that were microinjected into mouse eggs. The five linear DNA molecules that were microinjected are indicated in the upper diagram along with their 5′ end points. The solid boxes represent exons. The location of the 287-bp *Dde*I probe used to detect transgenic mice carrying human β-globin genes is indicated. The sequence of the 5′ end of human β-globin and mouse β^t-globin mRNA are shown in the lower diagram along with the 21-nucleotide oligomers that were used as specific probes to discriminate between these mRNAs. The differences in the oligomers are underlined.

that there is no cross-hybridization of this probe with mouse reticulocyte RNA and that the background is very low. The amount of globin mRNA can be calculated from the standard curve by knowing the size of globin mRNA and the M13 standard and by assuming that the probe hybridizes to both with the same efficiency. To calculate the number of mRNA molecules per cell one can normally use the amount of DNA in the sample, but because mouse reticulocytes lack DNA it was necessary to measure RNA. We determined that there is ~0.5 pg RNA per reticulocyte by measuring the RNA content of a known number of reticulocytes. The curves shown in Figure 2 for the two transgenic mice that expressed human β-globin genes correspond to ~5 and 185 molecules of human β-globin mRNA per cell.

To compensate for any systematic errors that might influence these calculations, we also measured total mouse β-globin mRNA in the same samples using an analogous 21-mer (see Figure 1) that can hybridize to the mouse β^t and β^s mRNAs that are expressed in SJL x C57 hybrid mice (Weaver *et al.*, 1981). These two mouse β-globin genes have identical sequence in the region corresponding to the oligomer (S.Weaver, personal communication). The ratio of β^s to β^t expression is 7:3, based on protein analysis (C.Wawrzyniak and R.Popp, personal communication). The ratio of human β-globin mRNA to mouse β-globin mRNA provides a comparative evaluation of how well the human β-globin genes are expressed in the mouse.

The average amount of mouse β-globin mRNA per reticulocyte was 1680 pg/μg RNA or 2190 molecules/cell (mean of the 36 samples shown in Table I). This value is about half of that calculated by Ross *et al.* (1974). Our calculation depends upon four values: the number of cells (~6 x 10^9 cells/ml blood), the percentage reticulocytes (~50%), the recovery of RNA (1.5 mg/ml blood) and the measurement of β-globin mRNA (~1680 pg/μg RNA). The first two values are in reasonable agreement with expectations. The recovery of RNA is higher than that reported by Ross *et al.* (1974) and comparable (on the basis of wet weight) with that obtained from other tissues, such as liver, that are active in protein synthesis. The discrepancy between our value and that determined by Ross *et al.* (1974) may reflect either the purity of the standards or differences in the hybridization characteristics of the probes to the standards. The low amount of β-globin mRNA per cell primarily reflects the fact that reticulocytes have only ~0.5 pg of total RNA per cell whereas most cells have much more. For example, liver cells have ~29 pg of RNA; thus, if the same fraction of liver RNA

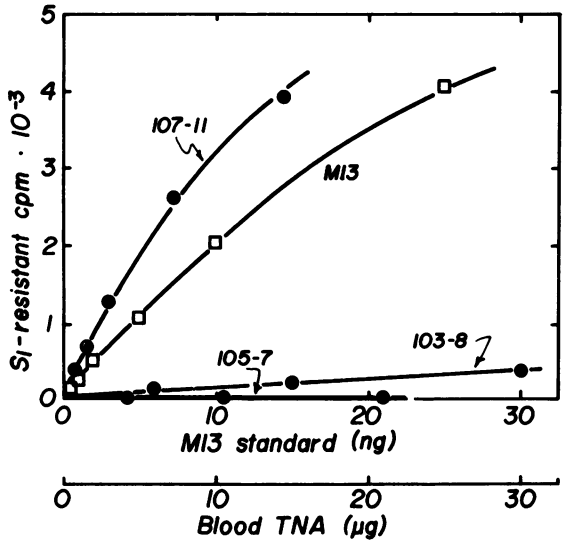

Fig. 2. Hybridization of the 21-mer complementary to human β-globin to the M13 standards and reticulocyte RNA from three mice (103-8, 105-7, and 107-11 indicated in Table I) bearing the −48 hβG construct. The amount of M13 single-stranded DNA or total nucleic acid (TNA) isolated from blood that was added to each hybridization reaction is shown. Total input of ^{32}P-labeled 21-mer was 8000 c.p.m. per reaction. The S1 nuclease-resistant radioactivity was measured as described in Materials and methods. No background has been subtracted. The M13 standard is ~8500 nucleotides whereas human β-globin mRNA is ~650 nucleotides long; therefore, 1000 pg of M13 is equivalent to 76 pg of human β-globin mRNA. The calculated amount of human β-globin mRNA in these samples is indicated in Table I.

Table I. Human β-globin gene expression in transgenic mice

DNA construct	Mouse number	hβ-globin genes/cell	hβ-globin mRNA[a]	mβ-globin mRNA[a]	hβG/mβG mRNA
Part A					
−4300 hβG	88-1	10	880	2600	0.337
	89-5	10	820	2080	0.394
−815 hβG	30-3	20	950	1380	0.688
	42-3	2	0	1510	
	42-4	3	45	1320	0.034
	42-6	2	0	1210	
	43-5	7	214	1630	0.131
	47-5	5	0	1110	
−360 hβG	116-3	1	29	1830	0.016
	120-2	5	207	1960	0.105
	122-1	3	237	1940	0.122
	123-9	2	0	2000	
1 122 hβG	109-3	20	237	1830	0.129
	109-4	3	126	1740	0.072
	109-5	9	17	1780	0.009
	113-2	25	557	1420	0.392
	114-5	20	1465	1490	0.983
−48 hβG	103-8	3	6	2080	0.003
	105-7	3	0	2080	
	107-11	30	144	1990	0.072
Part B					
−815 hβG	77-1	8	0	1590	
+ vector	77-7	3	0	1880	
	79-4	8	0	1450	
	80-1	13	0	1570	
	80-2	8	0.4	1500	0.0002
	81-2	8	0	1230	
	81-4	17	0	1620	
	81-5	2	0.6	1670	0.0003
	81-7	3	0	1880	
	82-2	4	0	1590	
	83-1	8	7.1	1640	0.004
	83-3	6	0	1500	
	84-5	8	0	1480	
	84-6	10	0	1690	
	84-8	3	0	1660	
	84-9	18	0	1610	

[a]Values for both human and mouse β-globin mRNA are pg mRNA/μg of total nucleic acid (almost all RNA) from blood of phenylhydrazine-treated mice; multiply values by 1.3 to convert to molecules per cell.

was β-globin mRNA, that would be equivalent to 127 000 molecules per cell.

Table IA summarizes the results of human β-globin gene expression in transgenic mice. Several mice in each group expressed human β-globin mRNA in blood cells although the level of expression varied considerably from mouse to mouse. The highest level of human β-globin gene expression was obtained in a mouse with the −122 hβG construct; the ratio of human β-globin mRNA to mouse β-globin mRNA (hβG/mβG) was 0.98, equivalent to ~1900 molecules of human β-globin mRNA per cell. There was at least one mouse in each group with >100 molecules of human β-globin mRNA per cell. The number of mice in each group is too low to know whether there is any relationship between the amount of 5′-flanking sequence and the level of expression. Nevertheless, note that two mice carrying human β-globin genes with only 48 bp of 5′-flanking sequence expressed these genes in blood cells (Figure 2 and Table IA).

We examined the DNA of several mice from each group to

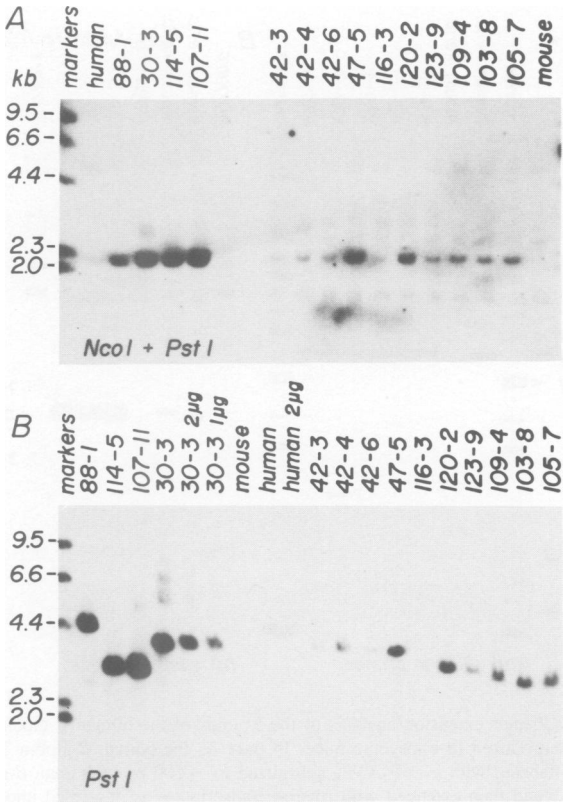

Fig. 3. Southern blot showing human β-globin genes in transgenic mice. Spleen DNA samples were prepared from the indicated transgenic mice (see Table I for identification) and digested with either *Nco*I and *Pst*I (**panel A**) or *Pst*I alone (**panel B**). The DNA was electrophoresed, blotted and hybridized with the 287-bp *Dde*I probe indicated in Figure 1 as described in Materials and methods. Human placental DNA and control mouse DNA samples were also included. 5 μg micrograms of DNA were loaded per lane except where indicated.

determine the gene copy number and to ascertain whether the genes from the mice that did not express any human β-globin mRNA were intact. Spleen DNA was digested with *Pst*I (Figure 3B) or *Pst*I and *Nco*I (Figure 3A) and the DNA analysed by agarose gel electrophoresis and Southern blotting. The blots were probed with the nick-translated fragment from the 3′-untranslated region (see Figure 1). Figure 3A, shows that the 2.1-kb *Nco*I-*Pst*I band corresponding to most of the β-globin gene was detected in all of the mice. The size of the *Pst*I bands in Figure 3B decreases in proportion to the removal of 5′-flanking sequence as would be expected if the DNA molecules were integrated as a tandem head-to-tail array. The data suggest that the human β-globin genes are intact even in those mice that do not express them. There is no apparent relationship between gene copy number and the amount of human β-globin mRNA produced. The lack of expression of foreign genes in some transgenic mice is a common finding (Palmiter *et al.*, 1983; Ornitz *et al.*, 1985) and probably represents integration into a region of chromatin that is incompatible with expression.

To examine whether human β-globin mRNAs start at the normal position, RNA from blood cells of one mouse from each set of 5′ deletions was hybridized with the oligonucleotide shown in Figure 1 and then extended by reverse transcription. Figure 4A shows that the major product synthesized was the same length as that produced from human reticulocyte RNA. Note that transcripts of the correct size were produced regardless of the amount of 5′-flanking sequence included with the human β-globin gene.

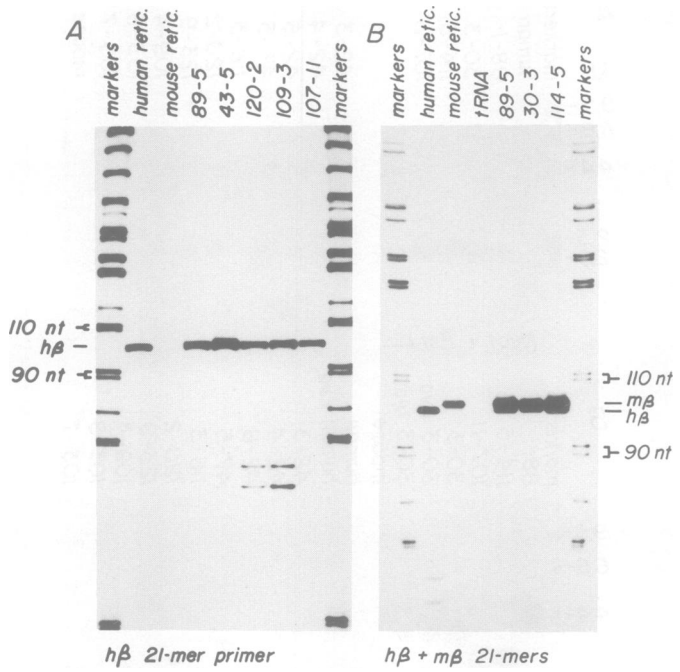

Fig. 4. Primer extension analysis of the 5′ ends of the human β-globin mRNA produced in transgenic mice. In **part A**, the human β-globin 21-mer was 5′-labeled with [γ-^{32}P]ATP, hybridized to ~500 pg of human β-globin mRNA, and then extended with reverse transcriptase as described under Materials and methods. The products were analysed by electrophoresis on an 8% polyacrylamide gel under denaturing conditions. **Lane 1**, end-labeled *Hpa*II markers prepared from pBR322; **lane 2**, human reticulocyte RNA obtained from a patient with sickle cell anemia (~500 pg of human β-globin mRNA); **lane 3**, control mouse reticulocyte RNA (0.5 μg); **lanes 4–8**, ~500 pg human β-globin mRNA from mouse 89-5 (−4300 hβG), 43-5 (−815 hβG), 120-2 (−360 hβG), 109-3 (−122 hβG) and 107-11 (−48 hβG), respectively; **lane 9**, *Hpa*II markers. In **part B**, equimolar amounts of the human and mouse β-globin 21-mers were 5′ end-labeled together, hybridized to the indicated RNA samples, extended with reverse transcriptase and electrophoresed on a 5% acrylamide gel as in **part A**. **Lane 1**, *Hpa*II markers; **lane 2**, human reticulocyte RNA; **lane 3**, control mouse reticulocyte RNA; **lane 4**, tRNA; **lane 5**, reticulocyte RNA from mouse 89-5 (−4300 hβG); **lane 6**, reticulocyte RNA from 30-3 (−815 hβG); **lane 7**, reticulocyte RNA from mouse 114-5 (−122 hβG); **lane 8**, *Hpa*II markers.

As a further check on the relative quantitation of human and mouse β-globin mRNA concentrations, RNA from blood samples from transgenic mice was primed with 21-mers specific for both mouse and human β-globin mRNA. Figure 1 shows that the product synthesized from mouse β-globin mRNA is slightly larger (100 nucleotides) than the product from human β-globin mRNA (98 nucleotides); thus, they can be distinguished when electrophoresed in the same lane. Blood RNA samples from several transgenic mice expressing significant amounts of human β-globin mRNA were primed with a 1:1 mixture of the two 21-mers that were kinased together, then elongated together by reverse transcriptase and electrophoresed under denaturing conditions. Figure 4B shows that a band that co-migrates in the position of human β-globin standard is seen just below the band which corresponds to mouse β-globin. The relative intensity of the band corresponding to human β-globin is only slightly less than the mouse β-globin band and the ratios appear consistent with the values obtained by solution hybridization (Table IA).

Procaryotic vector DNA interferes with human β-globin gene expression

In the experiments described above, the procaryotic plasmid sequences were removed prior to injection of the human β-globin genes. In another experiment, the pUC plasmid containing the β-globin gene with 815 bp of 5′-flanking sequences was only linearized at the 3′ end of the human DNA sequence prior to microinjection. Table IB shows that only three of 16 mice that retained this DNA expressed the human β-globin gene in blood cells. Moreover, the level of expression in these three mice was very low, <0.4% of mouse β-globin gene expression. Thus, it appears that DNA sequences present within this pUC vector interfere with expression of human β-globin genes in transgenic mice.

Table II. Ratio of human β-globin to mouse β-globin mRNA in different tissues of transgenic mice

Mouse	Globin	Tissue					
		Blood	Spleen	Brain	Liver	Kidney	Heart
		mRNA pg/μg total nucleic acid[a]					
30-3	hβ	950	80	8	12	12	39
	mβ	1380	125	9	15	15	40
43-5	hβ	210	19	<1	1	2	6
	mβ	1630	189	5	8	12	29
88-1	hβ	870	74	8			
	mβ	2610	270	26			
89-5	hβ	810	58	5			
	mβ	2080	190	13			
107-11	hβ	144	14	2			
	mβ	1990	156	18			
109-3	hβ	235	23	5			
	mβ	1830	161	31			
109-4	hβ	124	8	3			
	mβ	1740	151	30			
113-2	hβ	550	35	5			
	mβ	1420	88	10			
114-5	hβ	1450	93	14			
	mβ	1490	88	13			
120-2	hβ	204	15	1			
	mβ	1960	150	6			
122-1	hβ	235	16	1			
	mβ	1940	125	11			

[a]The amount of β-globin mRNA per cell was calculated as pg mRNA/μg total nucleic acid (TNA); these values can be converted to molecules per cell by multiplying by the following factors: blood, 1.3; spleen, 26; brain, 49; liver, 102; kidney, 36; and heart, 50.

Tissue specificity of human β-globin gene expression

As a first approach towards asking whether human β-globin gene expression is expressed in a tissue-specific manner, three different tissues (blood, spleen and brain) were examined from all of the mice and several additional tissues (liver, kidney, heart) were examined from some of the transgenic mice after phenylhydrazine treatment. Human β-globin mRNA was measured by the solution hybridization method and the values obtained are shown in Table II. None of the five mice that failed to express human β-globin mRNA in the blood expressed this mRNA in any other tissue that was examined (data not shown). In contrast, every mouse that expressed a significant amount of human β-globin mRNA in the blood also expressed measurable amounts in other tissues. However, the level of human β-globin mRNA in the other tissues (expressed as pg/μg total nucleic acid) was considerably lower than in blood. Because it seemed reasonable that some of human β-globin mRNA in other tissues could be due to contamination by circulating reticulocytes, we also measured mouse β-globin mRNA in these tissues (Table II).

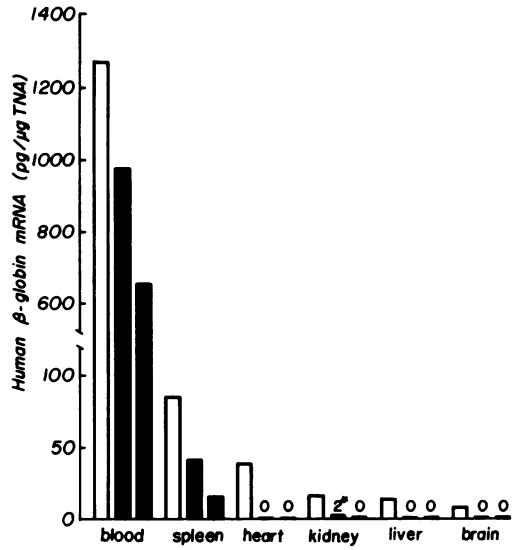

Fig. 5. Tissue specificity of human β-globin gene expression in offspring of 30-3, a transgenic mouse with the −815 hβG construct. Total nucleic acids were prepared from blood and the other tissues indicated and hybridized with the 21-mer specific for human β-globin as described in Materials and methods. One set of data was obtained from a 30-3 offspring made anemic with phenylhydrazine (open histograms); the other samples (solid histograms) were from two offspring of 30-3 that were not treated with phenylhydrazine but were perfused with saline after taking the blood sample to minimize contamination of the other tissues with blood. The kidney of one of the mice (*) did not perfuse well and retained some blood. The numbers over the histograms indicate the amount of mRNA detected; 0 signifies <0.1 pg/μg. The yield of total nucleic acids from blood of the two control mice was 16 and 5 fg per cell, compared with ~500 fg per cell for the phenylhydrazine-treated mouse.

Table III. Expression of human β-globin mRNA during development

Developmental stage	Globin mRNA (pg/μg RNA)		
	Mouse εY	Mouse β	Human β
11-day fetus	84	2	0
14-day fetal liver	30	220	28
Adult reticulocyte	0	2185	912

Hemizygous mouse 30-3 (−815 hβG) was outbred and then total nucleic acids were isolated from whole 11-day fetuses, 14-day fetal livers or from reticulocytes of phenylhydrazine-treated adults. Nucleic acids from those fetuses that were positive for the human β-globin gene were hybridized with oligonucleotides specific for mouse embryonic (εY) or adult (β) globin mRNA and for human β-globin mRNA as described in Materials and methods. Each value is the mean from two or three transgenic offspring; the values are corrected for RNA content but not for the fraction of total cells that express these genes.

In all cases, the ratios of human β-globin mRNA to mouse β-globin mRNA were similar in all of the tissues. Based on this analysis, we conclude that most, and perhaps all, of the human β-globin mRNA is produced in blood cells. However, low levels of expression of human β-globin mRNA in non-erythroid tissues would be undetectable due to the high background from circulating reticulocytes.

As a further check on tissue specificity, we examined the effect of phenylhydrazine treatment on human β-globin gene expression. We reasoned that if human β-globin mRNA is produced in erythroid cells, then the total amount of this mRNA in the blood should be significantly lower in mice that were not treated with phenylhydrazine because the number of reticulocytes would be substantially lower, but the specific activity (pg mRNA/μg

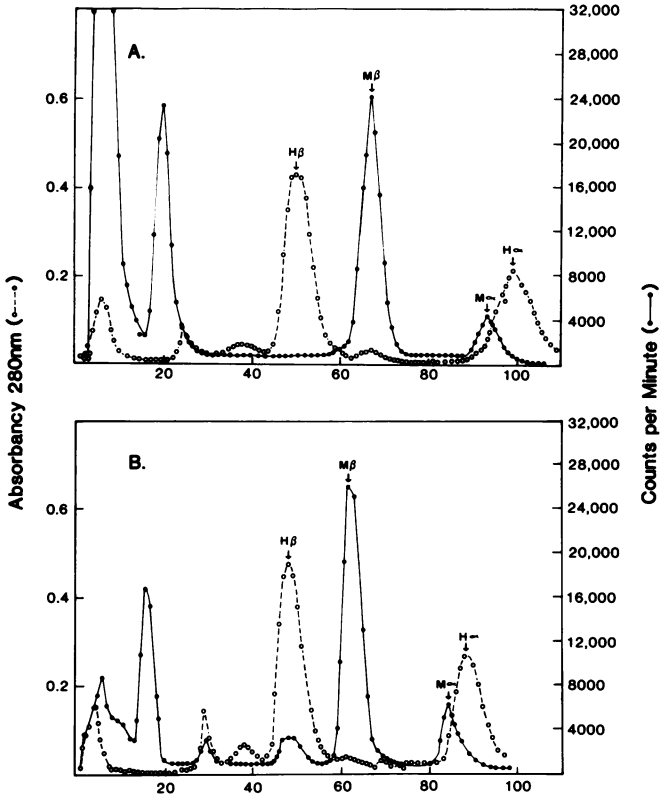

Fig. 6. Synthesis of human β-globin in lysates made from reticulocytes of transgenic mouse, 30-3. Offspring of mouse 30-3 (see Table I) were made anemic with phenylhydrazine, then reticulocyte lysates were prepared and incubated with [^{35}S]methionine under conditions optimal for protein synthesis. The reaction mixture was diluted into a hemolysate of human erythrocytes and the globin chains were precipitated with acid-acetone. They were applied to a CM-52 column and eluted with a salt gradient as described in Materials and methods. Aliquots were collected and the radioactivity and absorbance at 280 nm were determined. **A**, a typical profile from a control mouse; **B**, a typical profile from a transgenic offspring of 30-3 that expresses the −815 hβG gene.

total nucleic acid) should remain about the same since most of the nucleic acids in blood are derived from reticulocytes. Furthermore, by perfusing the mice with saline after drawing the blood samples, it should be possible to minimize the contamination of other tissues with blood. This experiment was performed on offspring from mouse 30-3 which carries the −815 hβG gene construct. Figure 5 shows that the specific activity of human β-globin mRNA in blood samples was only slightly lower (probably because of the relative enrichment of nucleic acids from circulating lymphoid cells) in two mice that were not treated with phenylhydrazine, but the total amount of nucleic acid recovered was 30- to 100-fold lower. In contrast, the combination of no phenylhydrazine treatment and perfusion eliminated human β-globin mRNA from all other tissues tested. These results are consistent with human β-globin mRNA being produced exclusively in erythroid cells in this line of mice.

A further implication of this experiment with offspring of mouse 30-3 is that the erythroid-specific expression of human β-globin genes can be transmitted faithfully to succeeding generations.

Developmental regulation of human β-globin gene expression

The timing of human β-globin gene expression during development was examined by analysing total nucleic acids isolated from

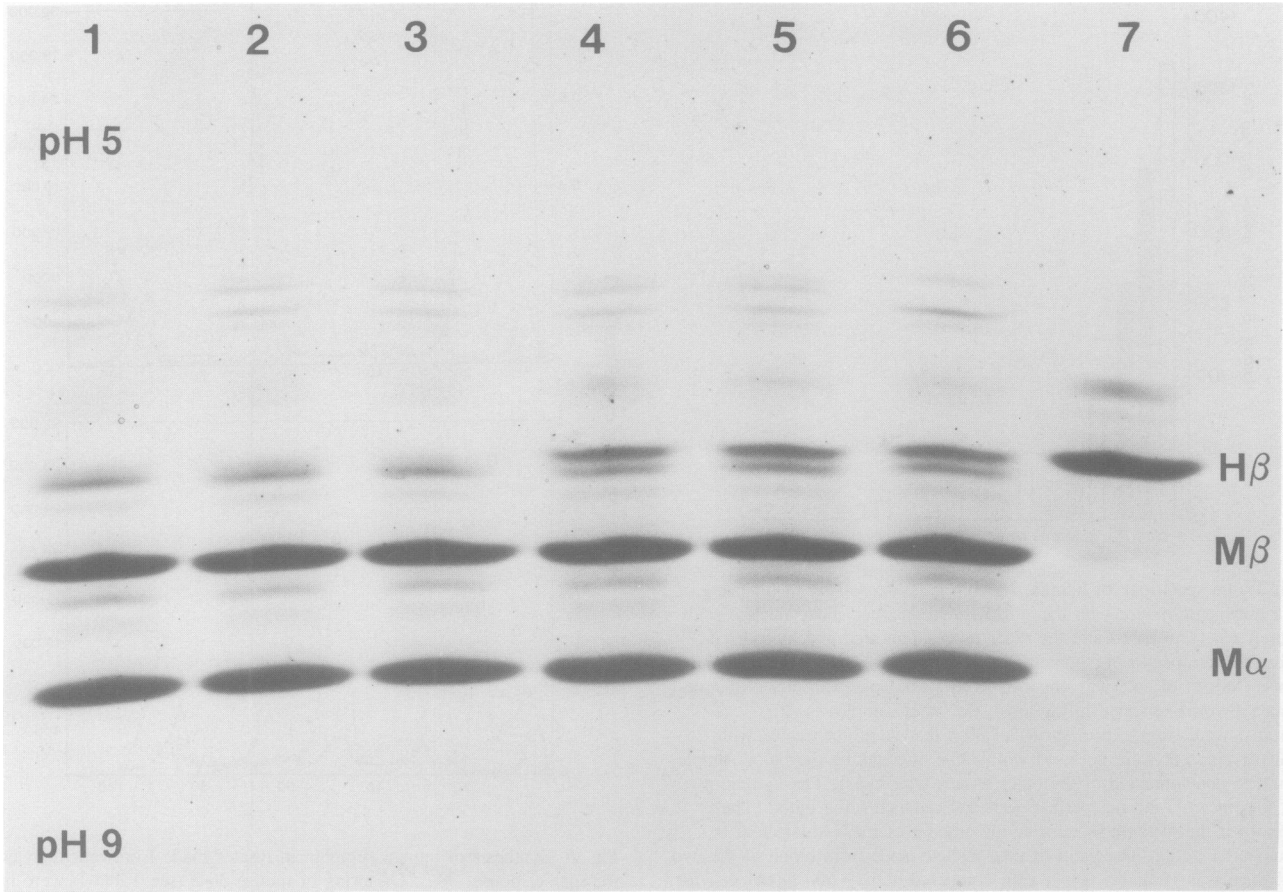

Fig. 7. Isoelectric focusing of globins obtained from control mice and transgenic offspring of 30-3. Globins were prepared, applied to an acrylamide gel, subjected to isoelectric focusing and the protein stained with Coomassie blue as described in Materials and methods. **Lanes 1–3** are samples from offspring of 30-3 that did not inherit the −815 hβG genes, **lanes 4–6** are samples from transgenic offspring of 30-3 and **lane 7** contains a sample of partially purified human β-globin The location of mouse α and β globin chains are indicated along with the location of the human β-globin markers.

11-day fetuses and 14-day fetal livers for human β-globin mRNA, mouse β-globin mRNA and mouse embryonic (εY) globin mRNA by solution hybridization (Table III). At 11 days, both human and mouse β-globin mRNAs were barely detectable but mouse embryonic β-globin was abundant. By 14 days, human and mouse β-globin mRNAs were detectable in fetal liver whereas mouse embryonic globin mRNA declined ~3-fold. In adult reticulocytes, mouse embryonic globin mRNA was undetectable, as expected, whereas human and mouse β-globin mRNAs continued to increase 10- to 30-fold. Thus, it appears that human and mouse β-globin genes are activated at about the same developmental stage.

Offspring of mouse 30-3 produce functional human β-globin mRNA and protein

To ascertain whether the human β-globin mRNA is functional, blood was drawn from anemic offspring of 30-3 and anemic control mice. Reticulocyte lysates were prepared and incubated with [^{35}S]methionine under conditions supporting protein synthesis. The radioactive products were mixed with unlabeled human hemoglobin, the globin chains were precipitated with acetone, dissolved and the α and β chains were applied to a carboxymethylcellulose (CM-52) column. A salt gradient was used to resolve the α- and β-globin chains. Figure 6A shows a typical profile from a control mouse. The absorbance at 280 nm marks the position of human β and α chains and the two major radioactive peaks mark the position of mouse β and α chains. The human and mouse β-globin chains are well resolved under this chromatographic system. Figure 6B shows a typical profile from a transgenic offspring of mouse 30-3. Note that there is an additional radioactive peak (centered on fraction 48) that is not present in the controls; it co-migrates with the human β-globin chains. The fraction of counts in the human β-globin peak relative to the mouse β-globin peak was 0.1 in this profile and it averaged 0.09 in four similar experiments. Considering the human β-globin chains have only one methionine residue whereas mouse β-globin chains have two, these results indicate that human β-globin synthesis represents ~18% of mouse β-globin synthesis in these mice. These results imply that human β-globin is functional in mouse reticulocyte lysates. However, the ratio of human β-globin to mouse β-globin synthesis is somewhat lower than the ratio of the two mRNAs, suggesting that human β-globin mRNA is perhaps not translated as efficiently as mouse β-globin mRNA.

To ask whether human β-globin chains persist in mature red cells, blood was drawn from untreated control mice and transgenic offspring of 30-3. Hemoglobin was prepared and the proteins were then analysed by isoelectric focusing. Figure 7 shows that in all three samples from 30-3 offspring there is an additional band (lanes 4–6) that is absent in the three control mice (lanes 1–3); this band migrates in the same position as the human β-globin standard (lane 7). The protein that migrates in the position of human β-globin appears to represent 10–20% of the mouse β-globin, an amount comparable with the relative rate of

human β-globin synthesis in reticulocyte lysates. These results indicate that the human β-globin mRNA is also translated *in vivo* and that the product accumulates in mature erythrocytes.

Because human β-globin persists in mouse erythocytes, blood samples from five transgenic offspring of 30-3 and five control mice were analysed for standard hematological parameters such as number of red blood cells, percent reticulocytes, hematocrit, and total hemoglobin. Although all of the parameters suggested slight anemia in the transgenic mice, the values were not statistically different in the transgenic and control mice.

Discussion

Our results indicate that human β-globin genes can be transferred into mice and expressed in an erythroid-specific manner at rates approaching those of endogenous mouse β-globin genes. The RNA transcripts from the foreign β-globin genes have correct 5′ ends and are functional in directing the synthesis of human β-globin in reticulocyte lysates made from these transgenic mice. Moreover, the human β-globin chains persist in mature erythrocytes. These genes are heritable in a Mendelian fashion and they are activated at about the same time (between 11 and 14 days of fetal development) as mouse β-globin genes.

Several previous reports have described the introduction of various β-globin genes into mice by similar techniques (Wagner *et al.*, 1981a; 1981b; Costantini and Lacy, 1981; Lacy *et al.*, 1983; Chada *et al.*, 1985). However, in most of those cases the foreign β-globin genes either were not expressed, were expressed in inappropriate tissues, or were expressed at a very low level. The poor expression of globin genes in some of these experiments may have been due to inclusion of procaryotic vector sequences. The effect of inclusion of the pUC plasmid in our construct −815 hβG is striking; both the frequency and the level of expression of the human β-globin genes is dramatically reduced when the plasmid sequences are present (Table I). The vector in some of the previous studies was phage λ (Costantini and Lacy, 1981); thus, this procaryotic DNA may have similar inhibitory effects. We have also noted inhibitory effects of plasmid DNA on expression of metallothionein-human growth hormone gene constructs when introduced into mice but not when transfected into tissue culture cells (Palmiter *et al.*, 1985). Wagner *et al.* (1981b) showed by immunological and isoelectric focusing techniques that rabbit β-globin was present in hemolysates from transgenic mice and indicated that their mice had elevated hematocrit and reticulocyte counts suggesting that they were mildly thalassemic. However, their identification of β-globin protein was equivocal and they did not analyse globin mRNA or tissue specificity of expression. Some of their mice were generated with globin genes that were free of vector sequences; however, it is impossible to decipher which rabbit β-globin gene constructs were present in the mice that expressed rabbit β-globin protein (Wagner *et al.*, 1981b). Thus, it is not clear whether they also observed an effect of plasmid sequences. More recently, Chada *et al.* (1985) have achieved tissue-specific expression of a hybrid mouse/human β-globin gene but the maximum level of expression was only 2% of mouse β-globin mRNA. They also noted more frequent expression of the hybrid gene when vector sequences were removed.

A number of genes are expressed in a tissue-specific manner when transferred into mice including immunoglobulin light (Brinster *et al.*, 1983) and heavy chain genes (Grosschedl *et al.*, 1984), elastase (Swift *et al.*, 1984), and alpha-fetoprotein (Krumlauf *et al.*, 1985). The DNA sequences responsible for tissue-specific expression in transgenic mice are most narrowly defined for the rat elastase gene where a 213-bp fragment that includes the promoter can be fused to the human growth hormone gene to direct its expression exclusively to acinar cells of the pancreas (Ornitz *et al.*, 1985). The erythroid-specific expression of the human β-globin gene was most rigorously demonstrated for the construct that retains 815 bp of 5′-flanking sequence and 1700 bp of 3′-flanking sequence (Figure 4); however, the data for the other constructs are consistent with erythroid-specific expression (Table II). Particularly suprising to us was the observation that a construct with only 48 bp of 5′-flanking sequence was still expressed reasonably well (~10% of mouse β-globin) in one mouse. Experiments involving 5′ and internal deletions indicate that removal of the tandemly repeated sequences in the −100 region and the CCAAT box in the −70 region severely depressed the level of expression of β-globin genes in transient assay systems (Grosveld *et al.*, 1982; Dierks *et al.*, 1983). However, Wright *et al.* (1984) showed that these regions were not essential when β-globin genes were stably introduced into murine erythroleukemia cells and the cells were induced to differentiate with hexamethylenebisacetamide; mutants with only 58 bp of 5′-flanking sequence were inducible. By testing a variety of fusion genes in this assay, Wright *et al.* (1984) and Charnay *et al.* (1984) concluded that the sequences responsive to erythroleukemia cell differentiation signals lie both 5′ and 3′ of the *Nco*I site at +53 (see Figure 1). They showed that sequences 3′ of this *Nco*I site are sufficient to increase transcription of the β-globin gene in response to inducers of erythroid differentiation. Thus, our results with the −48 hβG are consistent with their observations; in addition, our data indicate that the sequences that remain in the −48 hβG deletion are capable of responding to normal differentiation signals in an intact mouse. Because of the nature of the transgenic mouse assay and the small sample size, we cannot rule out the possibility that sequences upstream of −48 contribute to quantitatively normal β-globin gene expession.

Most globin genes that have been analysed show a strong DNase I-hypersensitive site located near the transcription start site (Stadler *et al.*, 1980; Groudine *et al.*, 1983). In normal human bone marrow cells, in which the β-globin genes are actively expressed, there is a prominent hypersensitive site located ~200 bp 5′ of the cap site; there are two additional sites located near the boundary of the second intron and third exon, and ~800 bp 3′ of the human β-globin gene (Groudine *et al.*, 1983). In only a few systems has the correlation between hypersensitive sites and transcriptional regulation been tested genetically. This correlation has been established in the case of the natural mutations that affect the hypersensitive sites and the level of expression of the *Sgs-4*, or glue protein gene of *Drosophila* (McGinnis *et al.*, 1983). Our results suggest that the DNA sequence underlying the hypersensitive site 5′ of the human β-globin gene is not essential for expression of this gene in erythroid cells of transgenic mice. It is possible that the DNA sequences responsible for the hypersensitive sites internal to and 3′ of the human β-globin gene are sufficient to allow appropriate expression of this gene in the absence of the 5′ sequences. Alternatively, it may be that the 5′ hypersensitive site is induced by sequences which lie within the gene; thus, the 5′ site may be generated in the appropriate place in the absence of the normal sequence that underlies it, as was recently demonstrated for a *Drosophila* hsp 70 hypersensitive site (Costlow *et al.*, 1985). Further studies are required to determine how hypersensitive sites are generated and whether they are primary correlates of gene determination.

Because of the wealth of information on human globin genes

and their proteins (Collins and Weissman, 1984), expression of these genes in animals should be particularly valuable. The system described here should allow access to many fundamental questions about cell-specific gene expression during development. In particular, this approach may allow insight into certain developmental mutants in which the level or timing of globin gene expression is inappropriate (Collins *et al.*, 1984; Gelinas *et al.*, 1985). It should now be possible to express genetically engineered or natural globin gene mutants as a means of understanding the regulatory mechanisms underlying gene commitment, RNA synthesis, translation and assembly of functional hemoglobin.

Materials and methods

Preparation of human β-globin genes for microinjection

A 10.8-kb *Xba*I fragment containing the human δ- β-globin genes was subcloned from λHβG1 (Lawn *et al.*, 1978) into pUC18. Human β-globin genes with 4300 and 815 bp of 5′-flanking sequence were isolated as *Taq*I-*Xba*I and *Hpa*I-*Xba*I fragments, respectively. A series of 5′ deletions were made with *Bal*31 exonuclease, an *Xba*I linker was inserted and the *Xba*I fragment containing the human β-globin gene was cloned into pUC18. The end points of the deletions were first estimated from acrylamide gels and then the −122 and −48 deletions were accurately determined by DNA sequencing. The appropriate DNA fragments containing the human β-globin gene were separated from other fragments on 1% agarose gels and isolated as described by Chen and Thomas (1980).

Microinjection and animal treatments

The DNA concentration was determined by a sensitive fluorescence method (Labarca and Paigen, 1980) and diluted to ~2 ng/μl for microinjection. The DNA was microinjected into the male pronuclei of F2 hybrid eggs of C57 x SJL parents as described by Brinster *et al.* (1985). Pups that retained the foreign DNA were detected by DNA dot hybridization to nucleic acids isolated from tail biopsies (Brinster *et al.*, 1985). Mice were made anemic by three injections (spaced ~12 h apart) of 0.4% phenylhydrazine solution (0.25 ml/25 g body weight) following the protocol of Cheng *et al.* (1974). Blood was drawn 5 days after starting the injections, reticulocyte counts were made (generally ~50% reticulocyte), and 0.1 ml of packed cells (~2 x 10^9 cells) were lysed in 4 ml SDS/proteinase K as described by Brinster *et al.* (1985). Portions (25−100 mg) of other tissues were homogenized in the same solution. Total nucleic acids (TNA) were then isolated by phenol/chloroform extraction and ethanol precipitation.

Solution hybridization

The procedure of Durnam and Palmiter (1983) was adapted for use with oligonucleotides. Total nucleic acids were dissolved in 0.2 x SET (1 x SET = 1% SDS, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5) and the concentrations of nucleic acids determined spectrophotometrically, assuming that an absorbance of 1 at 260 nm equals 50 μg/ml. Aliquots (10 μl with up to 40 μg TNA) were mixed with 20 μl of hybridization mixture containing 1 M NaCl, 33 mM Tris-HCl, 6.6 mM EDTA, pH 7.5, 0.2% SDS, and ~5−10 x 10^3 c.p.m. of ^{32}P-labeled oligonucleotide. Samples were overlaid with paraffin oil, incubated ~16 h at 45°C and then diluted with 1 ml of S1 buffer containing 100 μg of herring sperm DNA and 8 units of S1 nuclease, and incubated for 1 h at 37°C. The S1-resistant nucleic acids were precipitated with 100 μl of 6 M trichloroacetic acid for ~60 min at 0°C and then collected on glass fiber filters (Whatman GF/C) and counted in a Packard scintillation counter.

The sequences of the two β-globin oligonucleotides are shown in Figure 1; the εY 21-mer is 5′ ACCTCTTCAACATTGACCTTA 3′ and hybridizes to its mRNA between +12 and +123. Oligonucleotides were labeled by incubating ~10 pmol in a 10 μl reaction with 20 pmol [γ-^{32}P]ATP (~3000 Ci/mmol) and 1 unit of T4 kinase for ~30 min at 37°C. The labeled product was isolated on a 12% acrylamide gel and eluted in 1 ml of 0.2 x SET.

Each set of hybridizations included a standard curve with known amounts of single-stranded M13 DNA containing the strand complementary to the oligomer (see Figure 2). In the case of human β-globin, a 1200-bp *Sau*3A fragment spanning the first exon was cloned into mp18. For mouse β-globins, a 566-bp *Pst*I-*Bam*HI fragment spanning exon 1 of the β^t allele was cloned into mp9. A portion of the mouse embryonic εY gene was similarly cloned into M13. Single-stranded DNAs were prepared by standard procedures (Messing, 1983). The DNA concentration was determined by the fluorescence method (Labarca and Paigen, 1980) and multiplied by 2.5 to correct for single-stranded M13. This correction was determined empirically by comparing the values obtained by diphenylamine (Burton, 1968) and fluorescence methods.

DNA analysis

Spleen DNA was purified, digested with the indicated enzymes, aliquots (5 μg) were electrophoresed on a 1.0% agarose gel, and then the DNA was transferred to nitrocellulose by the method of Southern and hybridized with nick-translated probes as described by Palmiter *et al.* (1982).

Primer extension

Oligonucleotides were labeled and purified as described above. Approximately 0.05 pmol (200 000 d.p.m.) of the 5′ end-labeled oligonucleotide(s) were hybridized to total reticulocyte RNA for 30 min at 45°C in 50 μl of a solution containing 1.0 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS, and 20 μg of tRNA. After hybridization the samples were precipitated with ethanol, washed with 70% ethanol, dried, resuspended in 20 μl of a solution containing 50 mM Tris-HCl, pH 8.2, 6 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM NaCl, 1 mM of each of dTTP, dATP, dCTP and dGTP, 20 units of RNasin (Promega Biotec) and 15 units of avian reverse transcriptase (Boehringer-Mannheim) and the reaction mixture was incubated for 30 min at 37°C. Then reactions were extracted with phenol/chloroform, nucleic acids were precipitated with ethanol, dried, resuspended in denaturing buffer and electrophoresed on urea-acrylamide gels under denaturing conditions (Maxam and Gilbert, 1980).

Cell-free translation

The synthesis of β-globin chains was determined using reticulocyte lysates. Mice were made anemic by phenylhydrazine injection (Cheng *et al.*, 1974), reticulocytes were isolated and lysates prepared and incubated as described by Jackson and Hunt (1983) except that the lysates were not treated with nuclease. Each 100 μl reaction contained 90 μCi of [^{35}S]methionine (~1000 Ci/mmol) and was incubated for 10 min at 30°C. One half of the mixture was added to 10 mg of carrier human hemoglobin, globin was then prepared by acetone precipitation and the globin chains were separated using CM52 column chromatography (Lockard and Lingrel, 1972). The columns were 0.9 x 10 cm and the globin chains were eluted using a 160 ml linear gradient. The starting buffer was 10 mM Na_2HPO_4, pH 6.6, and the final buffer was 30 mM Na_2PO_4, pH 6.8. Both buffers contained 50 mM 2-mercaptoethanol and 8 M urea. Fractions (1 ml) were collected and the absorbance at 280 nm was determined. The radioactivity in aliquots (0.6 ml) was determined with a scintillation counter.

Analysis of globin proteins

The presence of human β-globin in transgenic mice was analysed by isoelectric focusing. Blood was drawn using heparin as an anti-coagulant and total globin was prepared by the acid-acetone procedure (Clegg *et al.*, 1966). Globin samples (100 μg) were dissolved in 8 M urea, 10% 2-mercaptoethanol and focused on 6% polyacrylamide slab gels (Awdeh *et al.*, 1968) containing 8 M urea, 2% Pharmalyte pH 6.5−9, 0.4% Pharmalyte pH 4−6.5 (Pharmacia Fine Chemicals), 5% glycerol and then stained with Coomassie Brilliant Blue.

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